ECA Foundation
Fostering harmonisation of GMP/GDP regulations

Bioburden – Regulatory Expectations and Experiences Questions & Answers

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Disclaimer

The questions in this document were compiled from the ECA Bioburden workshops held over the past three years and the answers were written in close cooperation with and to the best of the knowledge and belief of the experts who gave the presentations.

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1 Background

With the chapter <1115> "Bioburden Control of Nonsterile Drug Substances and Products", the USP brought the topic of bioburden into the focus of pharmaceutical manufacturers. The chapter concentrates on

- Increased emphasis on the control of microbial populations throughout the production cycle of excipients, active ingredients and finished medicinal products.
- Greater emphasis on the control of microbial populations at all stages of manufacture in the development of formulations and manufacturing processes.
- Place greater emphasis on water activity as a control measure.
- Greater emphasis on water for pharmaceutical use and cleaning of process equipment.
- Introduce content that raises awareness of housekeeping and disinfection as control measures.

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But "bioburden" is not just an issue for non-sterile products. Annex 1 of the European GMP Guide requires: "*Bioburden should be monitored prior to sterilisation. There should be working limits for contamination immediately prior to sterilisation that relate to the efficiency of the method to be used. The bioburden test should be performed for each batch, both for aseptically filled products and for terminally sterilised products*."

Last but not least, bioburden testing for medical devices manufactured or used in the US is regulated by Title 21 of the Code of Federal Regulations and globally by ISO 11737.
Recent developments and revisions prompted the ECA Academy to address this topic in a special workshop session to look at it from different angles and provide information on the regulatory background as well as practical examples and strategies for bioburden control. Pharmacopoeia experts, representatives of pharmaceutical quality control laboratories compiled the most important information and showed the challenges of bioburden control strategy and how to implement adequate control concepts in companies. From the questions that arose during this workshop, the experts started to compile a Q&A document.

2 Bioburden Testing

2.1 (i) "A limit of 10 CFU per 100 mL is generally accepted for bioburden before sterile filtration. Do you think that starting with a 100 mL sample and separately analyzing 50 mL on TSA medium and 50 mL on Sabouraud dextrose agar, then adding the two results, is an acceptable approach to provide a bioburden result for 100 mL? Are you aware of such an approach being used in the industry to reduce the amount of product used for QC testing?" (ii) "Do you also consider splitting the sample in two aliquotes: one on TSA for TAMC and one on SDA for TYMC and summing both results? Would it be acceptable? For example, for a 10 mL sample, 5 mL would be used for TAMC & 5 mL for TYMC; if we find 3 cfu for TAMC and 2 cfu for TYMC, the total would be 5 cfu /10 mL sample size?" (iii) "Sample amount for bioburden testing of APIs is usually 10g. Can the sample amount be reduced for cytotoxic APIs? Is there any need to start with this high amount? How this could be justified?"

For the membrane filtration method the description in Ph. Eur. 2.6.12 "Microbiological examination of non-sterile products: microbial enumeration tests" provides guidance in sections 5. TESTING OF PRODUCTS (see sub-section 5-1. AMOUNT USED FOR THE TEST and 5-2. EXAMINATION OF THE PRODUCT): "Unless otherwise prescribed, use 10 g or 10 mL of the product to be examined" and "[…] transfer the appropriate amount to each of 2 membrane filters and filter immediately. […] For the determination of TAMC, transfer one of the membrane filters to the surface of casein soya bean digest agar. For the determination of TYMC, transfer the other membrane to the surface of Sabouraud-dextrose agar."

This means that for a required or internally defined sample volume of 10 ml, 10 ml each must be tested for the TAMC and the TYMC. However, the Ph. Eur. describes the following exceptions: "For materials used as active substances where sample quantity is limited or batch size is extremely small (i.e. less than 1000 mL or 1000 g), the amount tested shall be 1 per cent of the batch unless a lesser amount is prescribed or justified and authorised. "For products where the total number of entities in a batch is less than 200 (e.g. samples

used in clinical trials), the sample size may be reduced to 2 units, or 1 unit if the size is less than 100." (see 5. TESTING OF PRODUCTS, 5-1. AMOUNT USED FOR THE TEST).
It is recommended that sample volumes deviating from the cited specifications should be discussed upfront with the competent competent authorities and approved by the authority.

In the case of the bioburden determination before sterile filtration, the "EMA-Guideline on the sterilization of the medicinal product, active substance, excipient and primary container" gives guidance regarding volumes to be tested. Here it is stated, that 100 ml, should be tested for TAMC, while TYMC is not mentioned. It is generally accepted that only TAMC is tested, if the recovery of representative yeasts and molds can be shown during method suitability. The draft USP chapter <1119> also hints in this direction. The testing volume of 100 mL may also be reduced, if justified.

2.2 Is the action limit and the specification limit the same thing? For example, it is appropiate to set the action limit for WFI as 10 CFU/100 mL or 0.25 EU/mL?

No – the definitions in ICH Q6B are as follows:

- **Specification**: A specification is defined as a list of tests, references to analytical procedures, and appropriate acceptance criteria which are numerical limits, ranges, or other criteria for the tests described. It establishes the set of criteria to which a drug substance, drug product or materials at other stages of its manufacture should conform to be considered acceptable for its intended use. "Conformance to specification" means that the drug substance and drug product, when tested according to the listed analytical procedures, will meet the acceptance criteria. Specifications are critical quality standards that are proposed and justified by the manufacturer and approved by regulatory authorities as conditions of approval.
- **Action limit**: An internal (in-house) value used to assess the consistency of the process at less critical steps.

2.3 With the "Proposed Compendial Approach for the Implementation of Microbiological Examination of Nonsterile Products–Tests for Burkholderia Cepacia Complex (BCC)" is there any clarification on the expectation for water testing? In the current version the wording 'optional testing of water' is vague.

No Ph. Eur. Eur. monograph - neither in the water monographs nor in a product monograph – requires to demonstrate the absence of *B. cepacia* or organisms of the BCC. However, for certain products or classes of products, a contamination with *B. cepacia* or organisms of the BCC may pose a risk to patients. However, this must be defined on a product or product class-specific basis. In these cases, the absence of *B. cepacia* or organisms of the BCC in the final product must be demonstrated.

2.4 Bioburden Test performance (slide 14): it was explained that the TSA plates are incubated 5-7days at 25-30°C, but the UPS draft chapter writes the standard TAMC incubation for 3-5 days at 30-35°C. what is your strategy/rationale?

Ph. Eur. 2.6.12 and USP<61> are harmonized via the PDG and the ICH-process. In both pharmacopeias the requirements are as follows: "Incubate the plate of Soybean–Casein Digest Agar at 30° to 35° for 3 to 5 days and the plate of Sabouraud Dextrose Agar at 20° to 25° for 5 to 7 days."

Note: Other incubation regimes are accepted, if appropriately justified and validated.

2.5 Do you have a rational supporting the use of TSA only for bioburden test (by opposition to Eur. Ph.2.6.12 prescribing TSA and SDA)

Data were generated that show that the TAMC is suitable for detecting a wide range of aerobic microorganisms, including yeasts and fungi. These data have been discussed with numerous regulatory

authorities worldwide. Based on these results, the regulatory authorities have approved the sole use of the TAMC for the microbial control of the biopharmaceutical production processes.

2.6 In practice, how do you manage a sample hold time exceedance? Do you generate data on microbial growth kinetics to support a risk assessment? If yes, how do you support the choice of microorganisms included in the study?

If a sample hold time is exceeded the sample is considered invalid, resulting in a "missing bioburden" event. If you are at risk of exceeding the hold time due to your planned process (e.g. external lab), you should validate a sufficient sample hold time upfront.

2.7 In Hold Time Consideration, do you also take into account the exposure to light?

No.

2.8 Do you validate a Cumulative Hold Time at large scale manufacturing?

No.

2.9 How many batches should be sampled for bioburden to establish production bulk holding time - e.g. holding time between start of compounding and end of 1st filtration?

Three batches.

2.10 *Regulatory expectations related to bioburden data integrity. Second verifier analyst requirement for plate counting?*

In theory, yes. For some bioburden sampling points the contemporaneous verification could be waived based on a risk assessment. Guidance for such a risk assessment provides BioPhorum's RISK ASSESSMENT OF TRADITIONAL CULTURE-BASED MICROBIOLOGICAL TESTS REQUIRING CONTEMPORANEOUS VERIFICATION. The current draft of the revision of USP <1117> follows this philosophy.

2.11 (i) "When the specification of the product has to be reported as combined TAMC and TYMC, should all colonies of each plate being counted, sum and reported regardless type of microorganism? I mean,
- TAMC: 1 bacteria and 1 Mold - 2 CFU/10 mL
- TYMC. 1 bacteria and 1 Mold - 2 CFU/10 mL
- Final result: Combined TAMC+TYMC: 4 CFU/10 mL?"

(ii) "What do you think about the sum of TYMC and TAMC results sometimes expected by EU inspectors?"

(iii) "You has TYMC in the past. How did you manage the results? TAMC and TYMC separatly compared to 10 CFU/100 ml? Or you made the sum of both (as expected by some authorities)?

Neither Roche nor Novartis perform a TYMC count. Hence, this aspect is irrelevant for us.

2.12 (i) "Is SDA required for monitoring each steps of the in the drug manufacturing process? OR TYMC specification is just required for product release?"
(ii) "Is TYMC really expected for in process bioburden testing for sterile products?"

Health authorities have accepted that we test all IPC levels and the Drug Substance using TAMC only.

2.13 (i) "What are the studies to perform to prove that TAMC is sufficient to recover the most probable MO in our environment?"
(ii) "Expose if they have develop tests to prove that most yeast and molds could be recovered on TSA in these incubation conditions?"
(iii) "Never any expectation to prove that any mold or yeast would grow on TSA in your incubation conditions?"

We did not perform any specific studies. Yeast and molds are included in our method suitability tests and these experiments and results were sufficient for the health authorities that we could waive the TYMC.

2.14 To recover yeast and molds on TSA should we modify the incubations conditions?

No - we incubate at 30 - 35 °C.

2.15 (i) "Did you somewhere demonstrate that the 24 hours shelf life of bioburden sample has no impact on the representativeness of the sample?"
(ii) "You said that the Bioburden Sample Hold Time of 24 hours was not always accepted. Could you share some examples?"

No - a shelf life of 24 hours was accepted by the health authorities without supporting experimental data.

2.16 (i) "For suitability / validation of IPC bioburden = how many local isolates used on how many different batches?"
(ii) "Which type and number of local strains do you add to the Growth Promotion Test?"

Two in-house isolates must be involved in the bioburden Method Suitability Test.

2.17 *Specify the incubation conditions for TAMC: temperature and duration?*

30-35°C for 3–5 days. Note: using a validated automated colony counter the incubation time is only 36 hours.

2.18 For bioburden filtration method, if no growth is detected, how the result should be reported as 0 CFU/10 mL or <1 CFU/10 mL for a sample with specification of ≤10 CFU/10mL?

You can report either 0 CFU/10 mL or <1 CFU/10 mL. Your internal test method must define the reporting.

2.19 Temperature 5°C is more important than time for bioburden...so what about an holding time of 48h max but keep at 5°C and without data? (note: explicit question to the Novartis colleague)

The sample hold time was discussed with numerous authorities and a 24-hour hold time was finally agreed upon. This sample hold time is described in the dossiers and accepted by the authorities.

2.20 Do you have retest samples for bioburden and how use it?

If the defined and filed shelf-life of the bioburden sample is exceeded (note: 24 hours) we do not have a valid sample anymore.

2.21 Hold time for DP release when sterility and LAL testing apply? 24 hours is requirement from sampling to testing, but is there any hold time restriction between filling and testing?

Typically, no sample hold time is defined for sterility test samples. An "endotoxin recovery study" should be performed to assess whether any sample-specific endotoxin masking effects requires defining a sample shelf life for endotoxin samples.

2.22 For phase III validation covering three batches (before commercial), can the IPC routine results be used for release when validation activities are ongoing? Or should the method validation covering all IPC steps be complete before routine IPC results can be used for release?

All validation efforts must be completed prior to the release of any material.

2.23 What is the rationale behind using In-house strains in Method Suitability Testing for Bioburden, which Sven (and others) mentioned? (note: explicit question to the Roche colleague)

The bioburden test method used should be able to detect microorganisms relevant for your manufacturing process and the respective facility / manufacturing environment. This will be demonstrated by including representative in-house isolates in your method suitability test.

3 Microbial Control System

3.1 (i) "What is the lower specification for a bioburden? A bioburden with specification less than 1 is not realistic? (due to the environment, consumables and equipment of the test)? So what should be the lower specification? Less than 3? Less than 5?" (ii) "Regarding bioburden limits: Samples from BDS production in Class D, C and B are taken aseptically and analyzed in a non-classified microbiological laboratory. Occasionally, a couple of colonies can appear on plates from sampling or handling. Does it make sense to have a bioburden limit of <1 CFU/10 mL in this setting or what would you recommend?"

A stepwise progression of limits is defined. Tighter limits are set closer to the end of the process with ≤ 10 CFU/10 mL if DS is frozen.

3.2 (i) "Did you perform a bioburden method suitability test for each defined sampling points? Or did you adopt an approach based on a worst case sample (probability of the intermediate / buffer to interferes with the test) to cover some other steps and reduce method suitability test effort based on a risk assessment?" (ii) "Authorities and inspectors - to what extend do they expect monitoring the control of

bioburden in the complete buffer/drug substance/drug product process, is this all risk based?"

Yes, a Method Suitability Test is performed for each defined sampling point. A risk-based approach is used to define the sampling points considering e.g. amongst others (i) configuration of process equipment, including the placement of bioburden reduction filters to avoid possible blind spots in detection of contaminants or (ii) open processing steps and surrounding environment or (iii) the potential impact of conditioning steps (e.g., extreme pH adjustments or solvent/detergent additions) for potential inactivation of putative bioburden must be considered or (iv) the growth-promoting capability of the process pool.

3.3 Should buffers which are received sterile filtered be tested for bioburden before being used in manufacturing? We already test for endotoxin.

There are no specifications that require this. If sterility of the buffer is mandatory, then this should also be verified by certificates or demonstrated by testing.

3.4 (i) "Should there be alert levels for all bioburden IPCs taken from aseptic production?" (ii) "For aseptic production in class C-D clean rooms, do you still recommend alert levels for all IPC steps? Or is alert levels for IPC steps only required when aseptic production in class A-B?"

Yes.

3.5 Can processes of pH manipulation of the pool (like viral inactivation) mask the presence of bioburden or endotoxins in it? I mean, is possible BB being detected in the BB analysis in a sample of the pool prior to execute the viral inactivation and not after the viral inactivation is ended?

Yes - the potential impact of conditioning steps (e.g., extreme pH adjustments or solvent/detergent additions) for potential inactivation of putative bioburden must be considered.

3.6 Do you always sample and test for endotoxins in parallel to Bioburden? If not why? (note: explicit question to the Novartis colleague)

Not always but in most cases. Note: EMA GUIDELINE ON THE STERILISATION OF THE MEDICINAL PRODUCT, ACTIVE SUBSTANCE, EXCIPIENT AND PRIMARY CONTAINER does not request to test for endotoxins prior to sterile filtration. Only a bioburden control is requested.

3.7 If alert levels are required for all IPC steps does it then apply to all product phases (Phase I-III, PPQ and commercial)? And how do you establish alert levels for phase I-III and PPQ if only a small amount of batches have been produced?

Yes. Provisional limits are used during development until sufficient historical data has been generated. Alternatively, for products manufactured infrequently (e.g. in development) data from similar processes may be used.

3.8 As the Bioburden is sampled before sterilization by filtration the product is not yet sterile so some inspectors want us to apply all expectations that are rather applicable to biological DS. Is this relevant? What would you recommend?

The philosophy of stepwise progression of the limits should be applied for DP manufacturing as well. Hence, the limits applied for Drug Product manufacturing should not be less stringent as the limits defined for Drug Substance. Stepwise progression of limits.

3.9 What about the bioburden (and its by-products) impact on used production equipment. In case of microbial counts; there is any guideline/rationale to assess resins/UFDF membranes safety to be used again in the manufacturing process? If the event is TNTC and no calculations can be performed, there is any way (e.g. some kind of blank run study) to defend no impact due to this byproduct?

Cleaning and sanitizing operations should be stringent enough to remove contaminants and any by-products. The effectiveness of the cleaning and sanitizing measures must be proven in a cleaning validation and a monitoring of the cleaning and sanitizing measures must be established in the routine.

3.10 Regarding hold time limits for manufacturing, is it important to define hold times for each process step? Or the overall hold time (all process steps until start of sterilization) is what matters from a microbiological point of view?

Yes. A maximum hold time for each process step must be defined and validated.

3.11 You described controls to assess the risk of contamination for Pharmaceutical ingredients and API manufacturers. To your knowledge, is there a requirement for environmental monitoring of facilities manufacturing API or raw material intended to be used for the manufacturing of non-sterile drug product (dry forms)

If a hygiene zone is defined for the production in which the production is to take place, then the requirements for this hygiene zone must be fulfilled.

4 Drug Substance Manufacturing

4.1 Is it bioburden anaerobic test required or FDA expected in any of the manufacturing process steps, such us in cell culture?

Yes, FDA requested to test for anaerobic bioburden at the end of the mammalian cell culture process.

4.2 Was the mammalian cell culture media used in the final bulk of the product?

No. In CHO-based expression systems, the product is released into the supernatant. The supernatant is purified. Hence, the cell culture medium is no longer in the final product.

4.3 Question: "What is the recommended method for pre-culture harvest samples in order to microbial detection, filtration method or liquid media as TSB?"

It depends on the specification for the pre-harvest samples. If "absence of any microbial contaminations" is defined, a qualitative method such as the direct inoculation could be used. If a bioburden limit is defined – e.g. ≤ 10 CFU/10 mL – a quantitative method must be used. Due to the defined volume to be tested, only the membrane filtration method is applicable.

4.4 Question: "Referring pre-filtration samples of the column eluate. Elution phase is usual not constant during the whole phase, normally a high peak of concentrated product is eluted in the first fraction of the elution while the concentration of product lowers as the end of the phase is nearing. Which moment should be sampled and how we can assure that is representative of the whole phase?"

The bioburden in the relevant fraction used for further processing should be tested.

5 Drug Product / Final Product Manufacturing

5.1 (i) "EMA Guideline on the sterilization of the medicinal product, active substance, excipient and primary container (6 march 2019) states that "In most situations, a limit of NMT 10 CFU/100 ml (TAMC) would be acceptable for bioburden testing". From your perspective, does therefore 100 ml represent the minimal volume to be filtered? And do you have experienced FDA expectation about minimal volume for product to be filtered?"
(ii) "What is a reasonable bioburden limit prior to sterilization of a small volume pharmaceutical solution? Guidelines suggest 100 cfu/100 ml, but if the whole batch is only one vial of 20 ml, this would mean 2 cfu per vial, almost sterile. 100 cfu/100 ml does not make sense, but what other limits may be justified?"
(iii) "Is the specification/acceptance level 10 CFU/100 ml?"

A sample volume of 100 mL is expected. Any deviation from this sample volume must be justified and accepted by the competent Health Authority. Generally, no "minimum" volume can be defined. It is always a case-by-case consideration.

5.2 Question: "For terminally sterilised products, for which the manufacturing process includes a bioburden reduction filtration step before sterilization, where in the process should IPC bioburden be sampled? Is it OK to test bioburden after filtration (but before sterilization)? Or bioburden should be tested on the bulk (before filtration and sterilization)?"

Additional sampling points may be required if the Drug Product manufacturing process includes further process steps.

5.3 (i) "When analyzing a 10 ml sample (IPC or DS), do you express final result in CFU/10 ml or do you allow extrapolation to 100 ml to comply with EMA Guideline on the sterilization of the medicinal product, active substance, excipient and primary container (6 march 2019)?"
(ii) "Spec < 10 CFU/100 ml ... if we have only 10 ml to be test, then I found not comfortable to have a specification < 1 CFU/10 mL : The bioburden test made under microbiological lab can't manage a specification of < 1 (equipment, environment of the test...)?"
(iii) "For small size batches you are ok to extrapolate a result obtained on a 10 ml (or 30 ml) sample to 100 ml. This is based on an assumption that micro contamination is homogeneous in a sample. However, most of time, germ are clustered in a sample. Therefore how do you justify you approach?"
(iv) "What is the considered a low batch size? max 10 liters ? more?"

Your internal test method must define the reporting. The final result could be reported in CFU/10 mL or after extrapolation in CFU/100 mL.

5.4 (i) "Did you demonstrate Mycoplasma retention capability of your 0.2 micron-filter when used for sterilization of products?" (ii) "Especially how did you manage USP<1229.17> chapter on mycoplasma sterilization? Or do you plan to go to a standard 0.1 micron sterilization?" (iii) "Bioburden prior sterile filtration: do you check really absence of Mycoplasma?"

Yes (for DS process) and No (for DP process). Typically, the requirements for filter used for final sterile filtration provided in sub-section 4.1.5. "Sterile filtration" and the filter data to be provided in the quality dossier is summarized in Table 3. of the EMA guideline are used. Absence of Mollicutes will be demonstrated at the end of the cell culture / USP process and aseptic manufacturing conditions guarantee that no Mollicutes contaminations occur during the DSP process. (Note: USP chapters above 1000 are not binding but for information only).

5.5 Alert level = how is perform the identification? Always by 16S rDNA sequencing?

Yes.

5.6 Annex 1: Terminally sterilisation stated as bioburden might be monitored. So not as mandatory. What is your experience with inspections/authorisations regarding this topic? Was that the reason to go to batchwise testing, or was there another reason?

EMA Guideline on the sterilisation of the medicinal product, active substance, excipient and primary container clearly requests monitoring of bioburden prior to the final sterilization process.

5.7 If the last Bioburden sample should be missed (due to lab error or other reason), would this immediately mean a rejection of batch, or could also then historical data save the batch? Or sterility testing of final product?

In principle, a missing bioburden result does not mean that the batch must be rejected. The company has the possibility to perform a risk assessment. If the bioburden result of a late IPC sample is missing - or even the bioburden result prior to the sterile filtration step - then it will probably be very difficult to release the batch on the basis of a risk assessment. Ultimately, the QP decides in Europe and the QA unit in other countries.

6 Risk Assessment

6.1 Is the risk assessment of MO recovered in an in-process bioburden for a sterile product an expectation?

During the batch release process, each contamination event must be investigated as to whether it could have an impact on the product and/or patient safety and thus whether the batch concerned can be released or not on the basis of a risk assessment.

6.2 Do you consider that the risk assessment documentation scope also include In-process control bioburden test on manufacturing intermediates or buffers (for biopharma drug substance manufacturing)?

Yes

6.3 (i) "Could you share some steps for the non-bioburden procedure if test for bioburden is missed."
(ii) "When a bioburden result is missed do you handle this situation as a non conform bioburden result?"

An Investigation of Missing Bioburden Result is a cross-functional activity including QC, QA and Production.

- In a first step, the reason for the missing bioburden result must be identified and CAPAs must be defined.
- The next step is to evaluate the ability of the manufacturing process to remove the contamination and/or possible byproducts.
  If available, the following data could be considered:
  - Bioburden results for the process steps before and after each sample type
  - Trend analysis and relevant bioburden results over the runs for the sample type in question
  - Endotoxin results for the first process step in which this test is performed after the sample type in question
  - Provide an assessment of environmental QC data for the production site and the Water used for process operations for the sample type in question.

6.4 Is patient safety and product quality assessment required for in-process test pre-fitration bioburden of sterile products?

No – the expectation is if the bioburden limit is exceeded the batch should be rejected.

6.5 For ID of bioburden >alert level, to what level to you perform identification (always to species-level, or Gram-characterization)?

16S rDNA sequencing is used as for the case-by-case assessment of bioburden excursion the contaminant must be identified to perform the next steps of the assessment.

6.6 Product Quality Impact (PQI) calculation. Is it taking into account the doubling time of the microorganism and the time from sampling until product pass through the filter to calculate the theoretical level of bioburden reached? And is this number use for the PQI calculation and not the bioburden count in the sample?

No, the bioburden count in the sample is the relevant value.

6.7 The critical components are analyzed in theory or really practical measures (with external contractors)?

It is a combination of literature review, experimental study (proteomic study) but also clinical studies.

6.8 Which stability tests are recommended for justified there is not PQI?

Assays that analyze the integrity of the product.

6.9 For the assessment of Bioburden Excursions, bacterial DNA and cellular components could potentially be released from all bacteria. This means that for these components the number of microorganisms in the IPC is used to calculate the risk (microbial ID does not matter in this case)?

For the fraction of e.g. flagellin or bacterial DNA we use generic values. However, for e.g. cell wall components, it makes a difference whether the contaminant is a Gram-positive or Gram-negative bacterium. Whether a contaminant produces exotoxins, and if so, which ones, can only be evaluated if the contaminant has been identified.

6.10 What are the key literature sources/references for the patient safety assessment? (note: explicit question to the Roche colleague)

An important document is the Registry of Toxic Effects of Chemical Substances (RTECS®), Comprehensive Guide to the RTECS, D. V. Sweet Ed., US Department of Health and Human Services, Public Health Service, 1997.

6.11 Literature search: do you find always the information about theoretical conc. of PAMPs?

Yes.

6.12 Proteases are evaluated during the PQI assessment right?

Yes.

6.13 If the result reported of the count is TNTC, is there any worst case to perform a PQI?

We do not use a generic assessment of bioburden excursions. The test method must be able to quantitate ≥ 10-fold the action limit. But theoretically, you could also do a calculation / an assessment assuming you have a contamination with 1000 CFU/mL or 10,000 CFU/mL of a worst-case microorganism. The model also works with such assumptions.

6.14 There is literature of more potent exotoxins with regards B. cereus, how do you determine *B. cereus* to be the worst case?

To our knowledge, botulinum toxins (produced by *Clostridium botulinum*) are the most potent bacterial toxins. Followed by tetanospasmin and tetanolysin (both from *Clostridium tetani*).

6.15 If the reduction of the bacterial byproducts cannot be measured, we don´t know if the theoretical values calculated are reduce in the following process steps. Why is stability only recommended if there is not further reduction steps?

We know from process validation that cellular components (e.g. host cell DNA, host cell proteins, ...) are removed during the downstream process. However, we cannot define exactly what the purification rate is for specific microbiological components. Therefore, we only perform the stability study if in final purification process steps the bioburden value exceeds our pre-defined limits.

6.16 Is it safe to calculate the risk for each component separately? Maybe two components that both induce innate immune responses have synergistic effects? Or this is covered by the worst-worst-worst case aspect of the calculations?

For each component the calculation is a "worst-worst-worst"-case assumption. Hence, we negate synergistic effects. This is also accepted by the health authorities.

7 Raw Material / Direct Material Testing

7.1 What about testing of raw materials that should be used for sterile prodcts, no testing of objectionable organisms is necessary for these raw materials?

For biopharmaceutical manufacturing processes no objectionable organisms are specified. Except for direct materials where a monograph exists. These direct material must fulfill pharmacopeial requirements – and this could include the test for specified microorganisms.

7.2 For bioburden testing of primary packaging such as flexible bags, is it necessary to perform extraction efficiency test and to test the empty packaging for bioburden? Or you can reason that the microorganisms that gets extracted into the product is what matters and that they will be detected in the bioburden testing of the product (if you test bioburden pre-sterilisation on filled products)?

No testing of sterile single-use disposables is required. The manufacturer can accept the vendor's certificate (if all GMP-requirements are fulfilled, e.g. Qualitiy Agreement exists, audits were performed, …).

7.3 Regarding Method Suitability Testing for Microbial Enumeration Tests when applied to Starting Materials. Should the hold time between adding the microbial suspension to the product solution and the filtration of the solution be quantified as a part of the MST? Should it be subjected to a Worst Case scenario or to an upper limit?

The method suitability test should reflect the bioburden testing under routine conditions. The method suitability test should be performed according to the relevant pharmacopeial General Chapter without any pre-incubation step after the addition of the spike microorganisms.